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Encapsidation studies of poliovirus subgenomic replicons

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The inclusion of a foreign marker gene, chloramphenicol acetyltransferase (CAT) gene, into the poliovirus genome allows its replication and encapsidation to be easily monitored using a simple enzyme assay. Such poliovirus replicons require the presence of helper virus for their successful propagation and thus are similar to defective interfering (DI) viruses. In genomes containing the CAT gene, the majority of the P1 virus capsid region of the poliovirus genome could be removed without destroying viability. The smallest replicon was significantly smaller than any naturally occurring DI particle so far reported, yet it retained the ability to replicate and be encapsidated by structural proteins provided by helper virus in *trans*. The efficiency with which the replicons were encapsidated was investigated using a direct immunostaining technique that allows individual cells infected with either a replicon or helper virus to be quantified. These results were compared to the frequencies of *trans*-encapsidation of polioviruses and coxsackievirus B4 using a two-stage neutralization assay. Poliovirus types 1, 2 and 3 but not coxsackievirus B4, coxsackievirus A21 or rhinovirus 14 provided efficient *trans*-encapsidation of poliovirus type 3 or type 3-derived replicons. These results suggest that a specific encapsidation process operates and that it does not involve RNA sequences within the region of the genome encoding the capsid proteins.

Introduction

Poliovirus contains a single-stranded positive-sense RNA genome of approximately 7450 nt bearing a small virus-encoded protein, VPg, covalently attached at the 5′ terminus and a poly(A) tract at the 3′ terminus. The RNA genome comprises a 5′ non-coding region (NCR) of about 740 nt, a large open reading frame encoding a virus polyprotein of M_r 220000 and a 3′ NCR of around 70 nt (Kitamura *et al.*, 1981; Racaniello & Baltimore, 1981; Stanway *et al.*, 1983). The virus polyprotein is divided into three regions based on primary cleavages. The P1 region contains the virus structural proteins and the P2 and P3 regions contain the non-structural proteins, which include two proteases and an RNA polymerase (Kitamura *et al.*, 1981). The highly conserved 5′ and 3′ NCRs of the genome are believed to have *cis*-acting functions that are essential for virus replication (Andino *et al.*, 1990; Rohll *et al.*, 1995, 1994*b*) and cap-independent translation (Pelletier & Sonenberg, 1989).

Genome replication and translation yields progeny genomes and capsid proteins that must be assembled to generate infectious virus particles. Although a variety of capsid assembly intermediates have been identified (Ansardi & Morrow, 1993; Jacobson & Baltimore, 1968; Nugent & Kirkegaard, 1995; Rombaut *et al.*, 1990; Yin, 1977), the mechanism by which the immature capsid proteins become associated with the genome is not understood. However, the encapsidation process is specific for positive-sense genomic viral RNA as it excludes other viral RNAs and cellular rRNA, tRNA or mRNA molecules (Nomoto *et al.*, 1977; Novak & Kirkegaard, 1991). The specificity for VPg-linked positive-sense viral RNA is likely to reflect the coupling of RNA synthesis to RNA encapsidation, rather than demonstrate a direct role for VPg in the encapsidation process; VPg-linked viral-sense genomes which accumulate in the presence of *p*-fluorophenylalanine (an inhibitor of virion formation) are not

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packaged after removal of the inhibitor (Harber *et al.*, 1991), and extensive modification of VPg has not lead to the identification of encapsidation mutants (Kuhn *et al.*, 1988; Wimmer *et al.*, 1993).

Cis-acting sequences that direct incorporation of the virus genome into progeny virions have been identified in a number of RNA viruses, including retroviruses, alphaviruses and coronaviruses (Schlesinger *et al.*, 1994). In many examples, the encapsidation signal consists of a discrete RNA secondary structure that, in certain cases, can be functionally transferred to a heterologous molecule.

Defective interfering particles (DIs) of many viruses have been studied as a means of identifying the minimum genomic structures which include all the *cis*-acting functions necessary for virus genome replication (Holland *et al.*, 1980; Perrault, 1981). Poliovirus DIs have been isolated from laboratory-propagated virus populations (Cole, 1975; Cole *et al.*, 1971; Kajigaya *et al.*, 1985; Lundquist *et al.*, 1979) and from manipulated cloned infectious cDNAs (Hagino Yamagishi & Nomoto, 1989) and, in all cases, retain translational as well as replication competence (Lundquist *et al.*, 1979; Omata *et al.*, 1986). The deletions carried by these DIs map to the P1 region of the genome (largely VP2 and/or VP3), and occupy between 4·2 and 13·2% of the genome size, which has been interpreted as indicating a minimum size of around 87% of the wild-type genome for RNA replication and encapsidation (Kajigaya *et al.*, 1985; Omata *et al.*, 1986). Since these replicons can be propagated in the presence of a homologous helper virus (that supplies capsid protein in *trans*) they must, by definition, retain an encapsidation signal. More extensive modifications of the P1 region can also be accommodated without abrogating replicon encapsidation. We have previously demonstrated that a poliovirus replicon (designated FLC/REP) carrying a chloramphenicol acetyltransferase (CAT) reporter gene in place of VP4 and VP2 (95% of the genome size) behaves similarly to a DI genome in that it is amplified following transfection of cells and is efficiently propagated by capsid proteins supplied in *trans* from helper virus (Percy *et al.*, 1992). We have suggested that the structure of naturally occurring DIs probably reflects the mechanism by which they are generated rather than indicating the minimal requirements for viability. They may, however, reflect a minimum size for packaging. Encapsidation of similar replicons has also been reported by other groups (Ansardi *et al.*, 1993; Porter *et al.*, 1993, 1995). Kaplan & Racaniello (1988) and Andino *et al.* (1993) described poliovirus RNA replicons lacking most of the P1 region, but did not report whether they could be encapsidated.

To further study the encapsidation of picornaviruses, we have constructed new poliovirus-derived replicons and investigated their complementation by a range of helper viruses. Although there are reports of heterologous *trans*-encapsidation of picornaviruses including poliovirus and coxsackievirus B1 (Cords & Holland, 1964 *a*, *b*; Holland & Cords, 1964), echovirus 7 and coxsackievirus A9 (Itoh & Melnick, 1959), and foot-and-mouth disease virus (FMDV) and bovine enterovirus (Trautman & Sutmoller, 1971), the methodology used did not permit quantification of *trans*-encapsidated genomes. We have therefore used an immunological assay to quantify heterologous *trans*-encapsidation of enteroviruses. Our results indicate that the encapsidation process is specific, and demonstrate that sequences within the P1 region of poliovirus are not involved.

Methods

■ **Construction of replicon cDNAs.** The construction and recovery of the CAT-containing subgenomic replicon FLC/REP from the cDNA pT7FLC/REP has been reported previously (Percy *et al.*, 1992). pT7FLC/REP2 and pT7FLC/REP3 were both derived from a full-length infectious molecular clone of poliovirus type 3 (pT7FLC). The CAT gene was amplified from pT7FLC/REP by PCR using the primers WSB63 (5′ TTTAAACTGCAGTAGCGCGCGGCGCACCTCCTTGCCATTCGTC 3′) and PIC3 (5′ GAATCTTCGACGCGTTGCGCTCAG 3′). WSB63 is complementary to the 3′ terminus of the CAT gene and introduces flanking *Bss*HII and *Pst*I restriction sites. PIC3 is positive sense and complementary to nt 269–292 of the poliovirus 5′ NCR. The purified PCR product was digested with *Sst*I and *Bss*HII and the 660 bp fragment cloned into *Sst*I/*Bss*HII-digested pT7FLC, replacing the 2·3 kb *Sst*I–*Bss*HII poliovirus fragment (nt 751–3295) and generating pT7FLC/REP3. pT7FLC/REP2 was constructed in essentially the same way. In all cases, the structures of the CAT/VP1 junction regions were verified by plasmid sequencing to ensure that an in-frame fusion was present.

The plasmid pT7FLC/REP4 contains a CAT gene replacing all but the last two amino acids of P1 and was constructed by amplifying the CAT gene with primers #643 (5′ TTGTTTGTTGGATCCATCGATATGGAGAAAAAAATC 3′; 5′ end of CAT gene) and #9 (5′ CCCAAAGCCATATGTCGCACCTCCTT 3′) to introduce *Bam*HI and *Nde*I restriction sites at the 5′ and 3′ ends, respectively. This fragment was subcloned in place of the 2·5 kb (nt 886–3373) pT7FLC to generate pT7FLC/REP4. It should be noted that due to the method of construction, nt 679–742 of the 5′ NCR of pT7FLC/REP4 are replaced with the sequence 5′ ATCGA 3′.

The coxsackievirus B4 (CB4) subgenomic replicon (CB4/REP) based upon an infectious molecular clone (Jenkins *et al.*, 1987; O. Jenkins, unpublished results) of CB4 was constructed. The resulting replicon carried a gene encoding CAT located between an *Sst*I site (introduced by site-directed mutagenesis at nt 748 in the region encoding the amino terminus of VP4) and an *Aat*II site (introduced by PCR mutagenesis at nt 1775 in the region encoding VP3).

■ **Construction of NCR recombinants.** Reciprocal substitution of the 5′ and 3′ NCRs of poliovirus type 3 Leon and CB4 was achieved using existing or introduced restriction sites and standard techniques (Sambrook *et al.*, 1989). An *Sst*I site was engineered into the region encoding the amino terminus of VP4 of an infectious cDNA clone of CB4 (O. Jenkins, unpublished results) allowing reciprocal exchange of this region with poliovirus type 3 Leon using the pre-existing *Sst*I site at nt 747. Transfection (see below) of the cDNA allowed the recovery of two recombinant viruses P5′CB4 and C5′PV3 (using a standard nomenclature where P5′CB4 indicates the 5′ NCR of poliovirus on an otherwise unaltered CB4 cDNA). Exchange of the 3′ NCR between the viruses was achieved following introduction of *Xba*I restriction sites at the translational stop codon of the polyproteins by gap-duplex mutagenesis using the oligonucleotides Leon *Xba*I (5′ CGACTGAGGTAGGGTTTCTAGAATGACTCAAGCC 3′) or CB4 *Xba*I (5′ GGTTGGATTCCTTCTAGATTAGAGACAATTTG 3′), resulting in the recombinant viruses P3′CB4 and C3′PV3.

In all cases, recovered virus was sequenced to confirm the presence of the authentic recombination, and the growth characteristics were shown to be comparable to those of the parental viruses by one-step growth curves (data not shown).

■ **Northern blot hybridization.** Tissue culture supernatant containing helper and subgenomic replicons was partially purified through a 30% sucrose cushion according to the method of Rico-Hesse *et al.* (1987); the RNA was extracted and hybridized using standard procedures (Sambrook *et al.*, 1989).

■ **Tissue culture, virus recovery and passage.** Ohio HeLa or Hep2C cells were grown and maintained as described previously (Minor, 1985). Replicon cDNAs were linearized with *Sal*I, used as templates for the *in vitro* synthesis of RNA runoffs by T7 RNA polymerase, and transfected into semi-confluent Ohio HeLa cells using DEAE–dextran, essentially as described previously (Evans & Almond, 1991). Where necessary, DNase-treated T7 transcripts were quantified using a GeneQuant (Pharmacia). Virus purification and plaque assays were carried out using the methods of Minor (1985). High multiplicity passage of subgenomic replicons involved the transfer of one-tenth volume (200 µl) supernatant to a fresh monolayer (10^6 cells). Where stated, the supernatant was treated with RNase A at a final concentration of 1 mg/ml.

■ **Immunostaining and CAT assays of subgenomic replicons.** The indirect immunostaining assay (blue cell assay) allows the identification of individual cells expressing virus or replicon-encoded antigens, and is based upon the method of Clapham *et al.* (1992). At fixed times post-infection (p.i.), the cell sheet was washed twice with PBS, fixed with ice-cold acetone/methanol (1:1 v/v) and washed once with 1% FCS/0·02% NaN_3 in PBS (wash buffer; WB). Primary monoclonal or polyclonal antisera were added at dilutions of 1:100 or 1:500, respectively, and incubated for 1 h at 4 °C before washing a further three times in WB. The *β*-galactosidase-conjugated secondary antibodies were added at a dilution of 1:100 and incubated for 1 h. After three further WB washes, the substrate (20 µg/ml X-Gal, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, 1·3 mM $MgCl_2$ in PBS) was added, the cells were incubated for 2–4 h at 37 °C and individual blue-stained cells were counted with an inverted microscope. Primary antibodies MAb204 (poliovirus type 3 VP1 site 1) and MAb700 (poliovirus type 1 VP2) were kindly supplied by M. Ferguson (NIBSC, Potters Bar, UK), polyclonal rabbit anti-CAT antibody was purchased from 5′-3′, and *β*-galactosidase-conjugated secondary antibodies (sheep *α*-mouse and donkey *α*-rabbit) were from Amersham. CAT activity expressed from the replicon reporter gene was determined as previously reported (Percy *et al.*, 1992), and scanned directly from the autoradiogram at 300 dots per inch prior to figure preparation in CorelDraw.

■ **Quantification of *trans*-encapsidation by neutralization assay.** *Trans*-encapsidation of poliovirus types 1 and 3 or CB4 and poliovirus type 3 was determined using a two-stage neutralization assay illustrated in Fig. 4. Monospecific rabbit antiserum to poliovirus type 3 Leon or CB4, or sheep antiserum to poliovirus type 1, were raised as described previously (Evans *et al.*, 1989). The cross-reactivity of the antisera was investigated by determining the minimum dilution of antiserum capable of neutralizing 10^7 p.f.u. of homologous virus without reducing the titre of 10^3 p.f.u. of heterologous virus in the same reaction. This was determined both for virus in suspension and when used in the overlay of a plaque assay. Quantification of the progeny virus populations from dual-infected Hep2C cells was determined using standard neutralization and plaque assay techniques (Minor, 1985; see also Results). Briefly, a monolayer of Hep2C cells were dually infected at an m.o.i of 10 p.f.u. per cell with each of two viruses. At 16 h p.i., the supernatant was harvested after a single freeze–thaw cycle and cell debris was removed by low-speed centrifugation. The supernatant was divided into two aliquots and virus in suspension was neutralized by the addition of antiserum specific for one of the two input viruses at the pre-determined dilution. Remaining infectivity was quantified by plating out the treated virus with an agar overlay containing either the same antiserum, or antiserum specific for the second virus in the dual infection. Plaques appearing in the latter dish represent *trans*-encapsidated genomes. This was confirmed by picking six plaques and demonstrating susceptibility to the primary neutralizing antiserum.

Results

Construction of CAT-containing replicons

Poliovirus subgenomic replicons constructed by the in-frame deletion of part of the P1 region have been reported previously (Choi *et al.*, 1991; Hagino Yamagishi & Nomoto, 1989; Kaplan & Racaniello, 1988; Percy *et al.*, 1992). We have demonstrated that one such replicon, FLC/REP, containing a CAT reporter gene, can be packaged into capsids supplied in *trans* by a helper poliovirus (Percy *et al.*, 1992).

To investigate whether replicons smaller than any naturally occurring DI genomes so far observed could be propagated in the presence of helper virus, three CAT-containing subgenomic replicons were constructed. All of these contained larger deletions in the P1 region than that in the previously described FLC/REP (Percy *et al.*, 1992) (see Fig. 1), and all are smaller than naturally occurring DIs. *In vitro* transcription of pT7FLC/

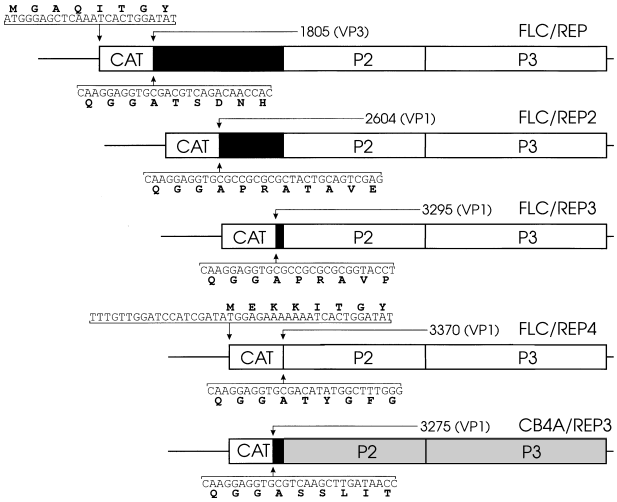

Fig. 1. Structure of poliovirus type 3 Leon- and CB4-derived subgenomic replicons. The construction of pT7FLC/REP has been described previously (Percy *et al.*, 1992). The cDNA sequence of the 5′ NCR/CAT junction is shown for FLC/REP and is identical in FLC/REP2 and FLC/REP3. The 5′ NCR/CAT junction of FLC/REP4 differs from that of the other replicons and is also indicated. The sequence across the CAT–P1 fusion is indicated below the diagram of each replicon. The truncated P1 region is shown as a filled box with the position (nucleotide number and capsid protein) of the fusion between CAT and P1 indicated above each diagram. The CB4-derived replicon (designated CB4A/REP3) is illustrated indicating the cDNA sequence at the junction of the in-frame fusion of CAT and VP1. The VP4/CAT in-frame fusion sequence is identical to that of the poliovirus Leon-derived replicons.

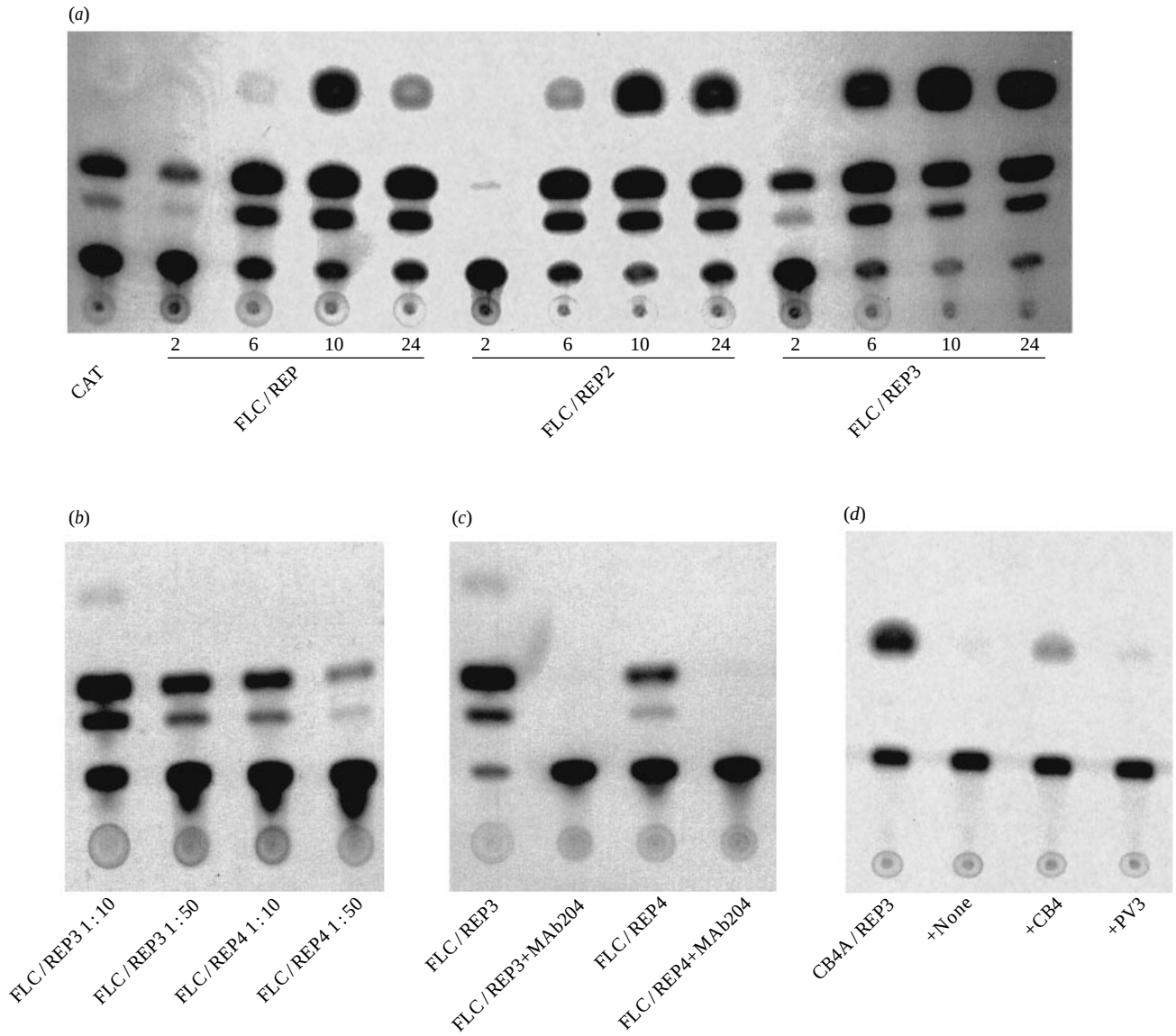

Fig. 2. Replication and encapsidation of poliovirus subgenomic replicons. CAT assays performed on replicon-transfected cells (*a* and *b*) and replicon-transfected cells subsequently superinfected with poliovirus type 3 helper virus. The CAT assays were performed on 50 μg cellular protein as described previously (Percy *et al.*, 1992). (*a*) CAT activity expressed by FLC/REP, FLC/REP2 and FLC/REP3 at 2, 6, 10 and 24 h post-transfection. CAT lane, activity from 1 U commercial CAT enzyme (Pharmacia). (*b*) Comparison of CAT activity expressed by FLC/REP3 and FLC/REP4 at 20 h post-transfection at the dilutions indicated. (*c*) Encapsidation of FLC/REP3 and FLC/REP4 by homologous helper virus. Cells transfected with FLC/REP3 or FLC/REP4 were infected 6 h post-transfection with poliovirus type 3 and harvested at 14 h p.i. After RNase treatment, the harvested virus population was transferred to a fresh monolayer either following incubation with or without the anti-poliovirus type 3 neutralizing antibody MAb204, and the transferred CAT activity was determined at 8 h p.i. from 50 μg cytoplasmic extract. (*d*) Replication and encapsidation of CB4A/REP3. Lanes: CB4A/REP3, replication at 6 h post-transfection; +None, CB4A/REP3 mock-encapsidated; +CB4, CB4A/REP3 encapsidated with CB4 helper virus; +PV3, CB4A/REP3 encapsidated with poliovirus type 3 Leon. The encapsidation and CAT assays were conducted as described in (*c*). These experiments used a monoacetylated CAT substrate.

REP2 produces an RNA of 6·2 kb (16% smaller than the full-length poliovirus RNA), pT7FLC/REP3 produces an RNA of 5·5 kb (25% smaller), and pT7FLC/REP4 generates a 5·4 kb RNA (27% smaller).

To determine whether the DI genomes were capable of efficient replication *in vivo*, T7 RNA transcripts were transfected onto monolayers of Ohio HeLa cells and cytoplasmic extracts were prepared at various times following transfection. The CAT activity produced from all the replicons increased with time, although the replication rates observed for FLC/REP4 were reduced when compared with FLC/REP3 (Fig. 2*a, b*). We have previously demonstrated that increasing CAT

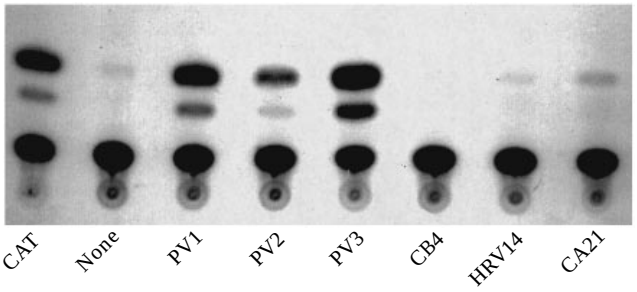

Fig. 3. *Trans*-encapsidation of FLC/REP by homologous and heterologous helper virus. Cells transfected with equal amounts of FLC/REP were infected 8 h post-transfection with a range of enteroviruses and rhinoviruses (at an m.o.i of 10) and harvested at 14 h p.i. Following RNase treatment, the recovered virus population was used to infect a fresh monolayer and the transferred CAT activity was determined at 8 h p.i. Lanes: CAT, 1 U CAT enzyme control; None, no helper virus; PV1–PV3, poliovirus types 1, 2 or 3, respectively; CB4, coxsackievirus type B4; HRV14, human rhinovirus type 14; CA21, coxsackievirus type A21.

activity reflects an increase in the amount of replicon RNA within the transfected cells (Percy *et al.*, 1992) and that this increase is sensitive to inhibition by guanidine.HCl (Rohll *et al.*, 1995).

A coxsackievirus-derived replicon, designated CB4A/REP3, which is essentially similar to FLC/REP3, was constructed and is shown in Fig. 1. PCR amplification and mutagenesis was used to replace nt 748–3275 with the CAT gene, and the in-frame fusion with VP4 and VP1 was confirmed by DNA sequencing. *In vitro* transcription of pT7CB4A/REP3 generates an RNA of 5·5 kb (25 % smaller than full-length CB4) which was shown to be replication-competent by the production of CAT activity following transfection of Ohio HeLa cells (data not shown).

Encapsidation of picornavirus subgenomic replicons

We next examined whether subgenomic replicon RNA could be encapsidated efficiently into poliovirus capsids provided in *trans*. The replicons were transfected into cells which were then superinfected with a homologous helper, poliovirus type 3, at an m.o.i of 1, as previously described (Percy *et al.*, 1992). After overnight incubation, the infected cell supernatants were treated with 1 mg/ml RNase A to remove any unencapsidated RNAs and used to infect fresh monolayers from which the CAT activity was determined at 8 h p.i. Fig. 2(*c*) shows that the CAT activities from FLC/REP3 and FLC/REP4 were propagated in the presence of helper virus in the same way as has been described for FLC/REP RNA. Similar results were obtained for FLC/REP2 (data not shown). Passage of the CAT activity was blocked by pre-incubating the encapsidated virus population with MAb204 specific for poliovirus type 3 capsids (Fig. 2*c*). CAT activity was only propagated when the replicon genomes replicated in the presence of helper virus and passage was inhibited by anti-poliovirus receptor MAb303 (data not shown), confirming that passage of the CAT-containing replicon was via poliovirus capsids and required the poliovirus receptor.

Specificity and efficiency of *trans*-encapsidation

***Trans*-encapsidation of subgenomic replicons.** We next determined whether poliovirus-derived CAT-containing replicons could be encapsidated by non-homologous helper viruses. FLC/REP was transfected into Ohio HeLa cells which were then superinfected with poliovirus types 1 or 2, human rhinovirus type 14 (HRV14), CB4 or coxsackievirus A21 (CA21). Infected cell supernatants were treated with RNase A and used to infect fresh monolayers, from which CAT activity was assayed (Fig. 3). Although there was some variation in the CAT signal obtained, significant levels of CAT activity were observed using poliovirus types 1, 2 or 3 as a helper virus. In the presence of heterologous helper viruses HRV14, CB4 or CA21, very low or undetectable levels of CAT activity were observed which could not be reduced by neutralization with antisera to the relevant helper virus, or anti-poliovirus receptor MAb303 (data not shown).

To confirm the specificity of the packaging process, we have also conducted the reciprocal experiment. A coxsackievirus-derived replicon essentially similar to FLC/REP3 was constructed (designated CB4A/REP3; Fig. 1) and encapsidation into capsids supplied in *trans* from coxsackievirus or poliovirus helpers was investigated. Using similar experimental conditions, transfer of CAT activity to a fresh cell sheet was achieved using a coxsackievirus, but not poliovirus type 3 Leon, helper (Fig. 2*d*). The specificity of encapsidation was confirmed by treating progeny virions with polyclonal serum to CB4 which blocked transfer of the CAT activity, encoded by CB4A/REP3, to the second cell sheet (data not shown).

The CAT signal obtained in these *trans*-encapsidation studies provides only an indirect quantification of the efficiency with which a replicon is encapsidated. We have therefore investigated this question further using an *in situ* immunostaining technique that allows the direct visualization and enumeration of virus-infected or CAT-expressing cells. This approach uses primary antibodies directed against either virus capsid proteins or CAT to detect infected cells. Bound antibodies are then detected using a suitable β-galactosidase-conjugated anti-species secondary antibody, the binding of which is visualized by the addition of X-Gal.

The sensitivity of this assay was confirmed by comparing the titre of a stock preparation of poliovirus type 3 Leon measured by plaque assay (stained and quantified at 3 days p.i.) and by *in situ* immunostaining at 8 h p.i. (with MAb204, specific for type 3 Leon VP1). In four independent assays, statistically similar results were generated, demonstrating that the techniques were equally sensitive. Using the *in situ* immunostaining assay, MAb700 (specific for poliovirus Sabin 1 VP2) or anti-CAT antibody enabled quantification of Sabin 1 and CAT-containing replicons, respectively, at 8 h p.i., but not

Table 1. Encapsidation of poliovirus replicons

(*a*) Factors that affect replicon *trans*-encapsidation frequency
Replicon RNA generated *in vitro* by T7 RNA polymerase was quantified and varying amounts transfected into subconfluent Ohio HeLa monolayers. Poliovirus type 3 Leon was added at 0, 4 or 8 h post-transfection at an m.o.i of 0·2, 2·0 or 10. Results are expressed as the ratio of replicon to helper virus numbers in the progeny population determined following treatment of the supernatant with RNase as described in the Methods. Helper virus or CAT-containing replicons were detected by *in situ* immunostaining assay using MAb204 or an anti-CAT antibody, respectively, and are the result of three independent assays.

Input replicon FLC/REP3 (μg)	Helper virus (m.o.i.)	Time post-transfection (h)	Ratio
0·74	0·2	8	1 in 11300
0·74	2·0	8	1 in 27700
0·34	0·2	8	1 in 9100
0·34	2·0	8	1 in 20800
1·50	10	4	1 in 370
1·50	10	0	1 in 680

(*b*) Serial passage of CAT-containing replicons and helper virus
The population generated from the encapsidation of 1·5 μg FLC/REP3 RNA with helper virus at an m.o.i. of 10 added at 4 h post-transfection (Table 1*a*, row 5) was serially passaged at high m.o.i onto a fresh cell sheet. The ratio of CAT-containing replicons in the encapsidated population was determined using the immunostaining assay used in Table 1(*a*). ND, Not determined.

Passage	CAT + cells	VP1 + cells	Ratio
1	$8{\cdot}2 \times 10^4$	$3{\cdot}0 \times 10^7$	1 in 370
2	$9{\cdot}2 \times 10^4$	$8{\cdot}2 \times 10^7$	1 in 900
3	$7{\cdot}4 \times 10^4$	ND	–
4	$1{\cdot}9 \times 10^5$	$2{\cdot}2 \times 10^8$	1 in 1100
5	$5{\cdot}9 \times 10^5$	$2{\cdot}8 \times 10^8$	1 in 480

at 0 h p.i. (even at high m.o.i.) demonstrating that detection requires *de novo* synthesis of virus proteins (data not shown).

We have investigated the effects upon encapsidation efficiency of different amounts of FLC/REP3 replicon RNA, helper virus and the time at which the encapsidated population is harvested. Efficiency was monitored by dividing the diluted cell supernatant and monitoring one half with anti-CAT and the other with MAb204 (anti-VP1) primary antibodies (Table 1*a*). Although only a small range of conditions were investigated, the results suggest that encapsidation efficiency is increased by transfection of the cell sheet with at least 1·5 μg replicon RNA per 10^6 cells, and the addition, optimally at 4 h post-transfection, of the helper virus at an m.o.i of 10 p.f.u. per cell (Table 1*a*). The FLC/REP3 replicon was present at a

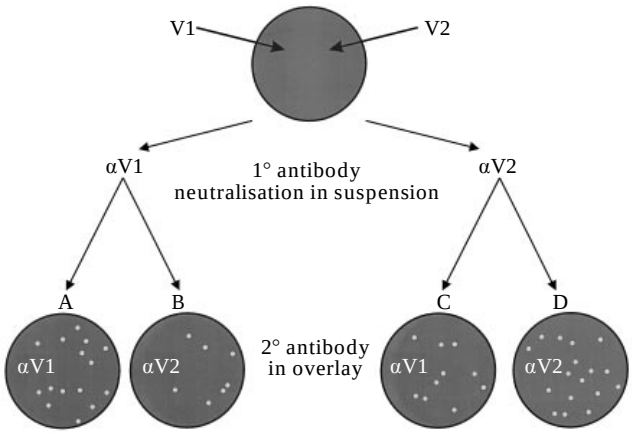

Fig. 4. Two-stage neutralization assay to determine the *trans*-encapsidation frequency of poliovirus. V1 and V2 represent the two virus types used in the study. The assay is described in the Methods. A–D represent the plaque assays used to determine residual virus remaining after neutralization with antisera αV1 or αV2, and are indicated in Table 2.

frequency of 1:370 under these conditions. Limited serial passage of this population of encapsidated replicon at a high m.o.i did not significantly alter the ratio of helper virus to replicon (Table 1*b*).

***Trans*-encapsidation of full-length genomes.** We have also investigated the frequency with which full-length virus genomes are *trans*-encapsidated using a two-stage neutralization assay. The assay is illustrated in Fig. 4 and utilizes mono-specific antiserum at pre-determined dilutions (see Methods). With this assay, it was possible to demonstrate that poliovirus types 1 and 3 *trans*-encapsidate at a frequency between 1:290 and 1:500 (Table 2*a*). Similarly, the *trans*-encapsidation of type 3 Leon and CB4 was measured at approximately 1 in 10^4 using an initial m.o.i of 10 p.f.u. per cell (Table 2*b*). *Trans*-encapsidation of CB4 and poliovirus type 3 was also tested at input m.o.i of up to 1000 p.f.u. per cell, which resulted in an approximately tenfold increase in the frequency (Table 2*b*).

We have previously demonstrated that replacement of the poliovirus 5′ or 3′ NCR with the corresponding region of CB4, or the reciprocal substitution onto a CB4 cDNA, yielded viable recombinant viruses whose single-step growth characteristics were indistinguishable from those of the parental viruses (D. Moon, unpublished results). The viability of these recombinant genomes means that they must contain an encapsidation signal. However, this does not exclude the possibility that the location of the signal in the two parental viruses may differ and therefore that the recombinants could contain two independent packaging signals. To formally test this hypothesis, we investigated the *trans*-encapsidation of 5′ or 3′ recombinants using the two-stage neutralization assay (Table 2*c*). *Trans*-encapsidation frequencies of between 1 in 8500 and 1 in 100000 were obtained, which are broadly in agreement with

Table 2. *Trans*-encapsidation ratios of poliovirus and CB4 as determined by a two-stage neutralization assay

The titre of virus remaining after neutralization (see Fig. 4) with primary (in suspension) and secondary (in plaque overlay) antisera are presented. Columns A, B, C and D show the data used to calculate the *trans*-encapsidation frequency given in the final two columns. Data are the average of three independent experiments.

(*a*) *Trans*-encapsidation of poliovirus types 1 and 3 (PV1 and PV3) at an m.o.i of 10.

		Primary antiserum …	α-PV3		α-PV1		*Trans*-encapsidation	
Virus	Total virus generated	Secondary antiserum …	α-PV3 A	α-PV1 B	α-PV1 C	α-PV3 D	PV1:PV3 A/B	PV3:PV1 C/D
PV1 + PV3	$2{\cdot}0 \times 10^8$		$0{\cdot}5 \times 10^8$	$1{\cdot}0 \times 10^5$	$4{\cdot}0 \times 10^6$	$1{\cdot}4 \times 10^4$	1 in 500	1 in 290
PV1	$0{\cdot}7 \times 10^8$		$0{\cdot}6 \times 10^8$	< 10	< 10	< 10	–	–
PV3	$0{\cdot}4 \times 10^8$		< 10	< 10	$8{\cdot}0 \times 10^6$	< 10	–	–

(*b*) Effects of increasing m.o.i. on *trans*-encapsidation of poliovirus type 1 and CB4.

		Primary antiserum …	α-PV3		α-CB4		*Trans*-encapsidation	
Virus m.o.i.	Total virus generated	Secondary antiserum …	α-PV3 A	α-CB4 B	α-CB4 C	α-PV3 D	CB4:PV3 A/B	PV3:CB4 C/D
10	$1{\cdot}6 \times 10^7$		$8{\cdot}0 \times 10^6$	$8{\cdot}0 \times 10^2$	$1{\cdot}6 \times 10^7$	$1{\cdot}7 \times 10^3$	1 in 9000	1 in 10000
100	$1{\cdot}6 \times 10^7$		$4{\cdot}8 \times 10^6$	$6{\cdot}0 \times 10^2$	$0{\cdot}6 \times 10^7$	$1{\cdot}2 \times 10^3$	1 in 5000	1 in 8000
500	$2{\cdot}0 \times 10^7$		$8{\cdot}0 \times 10^6$	$2{\cdot}4 \times 10^3$	$1{\cdot}0 \times 10^7$	$2{\cdot}8 \times 10^3$	1 in 3500	1 in 3300
1000	$0{\cdot}8 \times 10^7$		$6{\cdot}0 \times 10^6$	$4{\cdot}0 \times 10^3$	$0{\cdot}8 \times 10^7$	$6{\cdot}0 \times 10^3$	1 in 1300	1 in 1500

(*c*) *Trans*-encapsidation of poliovirus type 3 and CB4 chimeric viruses at an m.o.i. of 10.

		Primary antiserum …	α-PV3		α-CB4		*Trans*-encapsidation	
Virus	Total virus generated	Secondary antiserum …	α-PV3 A	α-CB4 B	α-CB4 C	α-PV3 D	CB4:PV3 A/B	PV3:CB4 C/D
PV3 + P5′CB4	$2{\cdot}0 \times 10^8$		$4{\cdot}0 \times 10^5$	$2{\cdot}0 \times 10^1$	$2{\cdot}0 \times 10^7$	$2{\cdot}0 \times 10^2$	1 in 20000	1 in 100000
CB4 + C5′PV3	$1{\cdot}3 \times 10^8$		$9{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^1$	$2{\cdot}5 \times 10^6$	$2{\cdot}0 \times 10^2$	1 in 18000	1 in 12500
P5′CB4 + C5′PV3	$1{\cdot}8 \times 10^8$		$8{\cdot}0 \times 10^5$	$3{\cdot}0 \times 10^1$	$6{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^2$	1 in 27000	1 in 20000
PV3 + P3′CB4	$4{\cdot}0 \times 10^7$		$5{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^1$	$5{\cdot}0 \times 10^6$	$6{\cdot}0 \times 10^2$	1 in 50000	1 in 8500
CB4 + C3′PV3	$5{\cdot}0 \times 10^7$		$8{\cdot}0 \times 10^5$	$2{\cdot}0 \times 10^1$	$2{\cdot}0 \times 10^6$	$1{\cdot}8 \times 10^2$	1 in 40000	1 in 11000
P3′CB4 + C3′PV3	$5{\cdot}0 \times 10^7$		$5{\cdot}0 \times 10^5$	$2{\cdot}0 \times 10^1$	$4{\cdot}0 \times 10^6$	$1{\cdot}9 \times 10^2$	1 in 25000	1 in 21000

those obtained at the same m.o.i (10) using unmodified poliovirus type 3 Leon and CB4 (see Table 2*b*).

Discussion

Naturally occurring DIs of poliovirus contain in-frame deletions within the P1 region of the genome (Cole, 1975; Kajigaya *et al.*, 1985; Lundquist *et al.*, 1979). We have previously suggested that the retention of VP4 in DIs probably reflects their mechanism of generation, rather than there being a functional requirement for sequences from this region of the genome to be included. This is because CAT-containing subgenomic replicons lacking VP4 can replicate efficiently and be encapsidated by homologous helper viruses (Percy *et al.*, 1992). We have extended these studies to determine the requirement for sequences within VP3 and VP1, and also investigated the efficiency and specificity of encapsidation.

The deletion carried by the original replicon (FLC/REP; 5% smaller than the poliovirus type 3 genome) was extended to 17, 25 and 27% during the construction of FLC/REP2, FLC/REP3 and FLC/REP4, respectively. The replication of the subgenomic replicons was demonstrated by transfection of cell

monolayers with T7 runoff RNAs generated *in vitro* from the cDNA. Although not precisely quantified, the replication rate of FLC/REP, FLC/REP2 and FLC/REP3 was approximately inversely proportional to the size of the replicon since expression of FLC/REP3 RNA resulted in the highest CAT activity. This is in agreement with a previous report in which the smallest of several subgenomic replicons (deleted by 1295 nt or 17% of the genome) replicated at a rate 1·4 times that of the wild-type genome (Kaplan & Racaniello, 1988), and further suggests that, although DI genomes replicate at a faster rate, the effect is modest for small deletions. The replication of FLC/REP4 was reduced by approximately fivefold in comparison with FLC/REP3 (Fig. 2*b*). This is possibly a consequence of the juxtaposition of the carboxyl terminus of CAT with the 2A protease cleavage site. Although not formally tested in a study of 2A cleavage specificity (Hellen *et al*., 1992), we note that the P4 leucine residue, conserved in both the P1-2A and P3C′-D′ cleavage sites of all poliovirus strains, is substituted for an alanine in this replicon. It is unlikely that the 5′ NCR modifications introduced during the construction of pT7FLC/REP4 are responsible for the reduced replication as others have already shown that the region immediately preceding the initiation codon can be deleted without deleterious effect (Kuge & Nomoto, 1987).

All three subgenomic replicons could be packaged into capsids supplied in *trans* by poliovirus type 3. Passage of CAT activity to a fresh cell monolayer was mediated by poliovirus capsids interacting with the poliovirus receptor as it was blocked by antibodies against either. FLC/REP could also be packaged by capsids supplied in *trans* by poliovirus types 1 and 2, but not by HRV14 or CB4. Similar results have been obtained with FLC/REP2 and FLC/REP3 (data not shown). Since the latter replicons exhibit an enhanced level of replication to FLC/REP (see Fig. 2*a*), it is likely that any specific and significant level of *trans*-encapsidation by a heterologous helper virus would be detectable in this assay.

These results indicate that there is specificity in the packaging of the poliovirus genome and suggest the existence of a specific encapsidation signal in the RNA as has been observed for other viruses. The precise location of such a signal remains to be elucidated. However, the observation that replicons (FLC/REP4) lacking all but the last two amino acids of the P1 region (nt 742–3370) could be encapsidated demonstrates that the signal for encapsidation does not reside within this region. Furthermore, recent data has shown that substitution of the CB4 2A protein for that of poliovirus type 1, or the generation of a hybrid coxsackievirus B3/poliovirus 2B protein were both compatible with virus viability, suggesting that a specific encapsidation signal does not occupy the region of the genome encoding either 2A or the first 30 residues of 2B (Lu *et al*., 1995; VanKuppeveld *et al*., 1997). We have demonstrated that NCR recombinant viruses constructed between poliovirus and CB4 are viable and do not contain additional packaging determinants that increase the frequency with which they can be encapsidated by a heterologous (with respect to the region of the genome encoding the polyprotein) helper virus. This demonstrates that the 5′ and 3′ NCRs alone do not contain the packaging determinants for either of these viruses and that, if they are involved in the encapsidation process, their removal is not deleterious to the virus. These results are in agreement with data from our laboratory that demonstrate that the poliovirus 3′ NCR can be substituted for the corresponding region of HRV14 without loss of virus viability (Rohll *et al*., 1994*a*, *b*), and that the entire poliovirus 5′ NCR can be exchanged for the corresponding region of rhinovirus type 2 (Xiang *et al*., 1995), or the internal ribosome entry site (IRES) element substituted for the corresponding regions of encephalomyocarditis virus or hepatitis C virus (Lu & Wimmer, 1996; Rohll *et al*., 1994*b*). These observations suggest that if an encapsidation signal exists, it is most likely to reside within the region of the genome encoding P2B to P3D. Furthermore, we have made reciprocal substitutions of the poliovirus type 3 Leon and CB4 VPg-encoding sequences in CAT-expressing subgenomic replicons, without observing any defect in encapsidation into capsids supplied by homologous helpers, or increases in encapsidation by heterologous helper viruses (N. Percy & D. Moon, unpublished data). These results support the conclusions drawn from previous studies; any *de facto* requirement for VPg in the encapsidation process is likely to reflect the intimate link between replication (in which VPg has a defined role) and packaging, rather than provide direct evidence that encapsidation is controlled by an interaction of the genome-linked VPg and capsid proteins (Harber *et al*., 1991; Kuhn *et al*., 1988; Wimmer *et al*., 1993).

The fact that replicons and DIs can be encapsidated at all suggests that capsids are able to transfer between replication complexes. The frequency of *trans*-encapsidation must be related to some extent to the efficiency with which replication complexes mix, as well as to the specificity of the encapsidation mechanism. We have described two independent assays to quantify the *trans*-encapsidation frequency of poliovirus. Although not directly comparable – one assay is based upon co-infection and the other upon transfection and infection – the results obtained suggest that the efficiency of homologous encapsidation is about two orders of magnitude greater than that using a heterologous helper virus. Increasing the m.o.i. to 1000 p.f.u. per cell increased the *trans*-encapsidation frequency of poliovirus type 3 and CB4 tenfold, which may possibly be due to an increase in the local concentration of replication complexes.

The specificity of *trans*-encapsidation may be due to compartmentalization, and hence inefficient mixing, of replication complexes within the cell, or to a requirement for defined interactions between the genome and capsid protein(s). The studies we have conducted address the role of specific regions of the genome in the encapsidation process. However, the recent report of the association of FMDV replication complex proteins with virus particles (Newman & Brown,

1997) adds an additional layer of complexity to our understanding of the components that may be involved in the encapsidation mechanism of picornaviruses, and the relative contribution of both protein and RNA components to picornavirus packaging remains to be determined.

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